Supporting Information

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SI Materials and Methods

Generation of Optimized Linker Sequences. Random DNA sequences of defined base composition were generated using the FaBox online toolbox (33). Melting temperatures were predicted using Thermo Scientific's Multiple Primer Analyzer tool for Phusion polymerase. Single-stranded DNA secondary structure formation probabilities were estimated using RNAfold (v2.0.7; parameters, –noGU –noPS -T 50; ViennaRNA Package) (34) and Zipfold (parameters, DNA, 50 °C, 0.1 M Na^+, 0.01 M Mg^{2+}; DINAMelt Web Server) (35, 36). Minimum free energy hybrid structures were predicted using RNAcofold (v2.0.7; parameters, –noGU –noPS -T 50; ViennaRNA Package) (34). The optimized sequence set was determined using a python script.

biGBac Vector Generation. The biGBac vectors described here are derived from the Multibac pFL vector (4, 10). The pLIB vector was generated by deletion of the p10 expression cassette from pFL by digestion with PmeI and BstZ17I followed by blunt end ligation (Fig. S2). The pBIG1 vectors were generated from pFL by replacing the p10 expression cassette, the multiplication module, and the polh expression cassette with the optimized DNA linker sequences for both assembly steps separated by spacer sequences of about 150 bp (derived from HSVtk terminator sequence in pFL) that contain SwaI, PmeI, and PacI endonuclease recognition sites (Fig. S3) using Gibson assembly reactions. In addition, the gene conferring spectinomycin resistance was inserted between the ampicillin resistance gene and the Tn7L sequence. The pBIG2 vectors were generated from pFL by replacing the p10 expression cassette, the multiplication module, and the polh expression cassette with DNA linker sequences and recognition sites of the endonucleases PmeI and PacI (Fig. S4) using Gibson assembly reactions. The gene conferring chloramphenicol resistance was inserted between the ampicillin resistance gene and the Tn7L sequence.

Baculovirus Generation and Protein Expression. pLIB, pBIG1, or pBIG2 constructs were used to generate recombinant baculoviral genomes by Tn7 transposition in DH10EMBacY cells (5). Viruses were generated by transfection of Sf9 insect cells (Thermo Scientific) with the recombinant baculoviral genome using Fugene 6 reagent (Promega). After 3 d, viruses were amplified by adding transfection supernatant to Sf9 suspension cultures. Protein complexes were expressed in Sf9 or HighFive (Thermo Scientific) insect cell suspension cultures.

Protein Purification. Recombinant APC/C and APC/C–CDH1–EMI1–SKP1 were expressed using the baculovirus expression system in HighFive cells (Thermo Scientific) and purified for biochemical analysis or EM essentially as described (8). In brief, APC/C, which is expressed with a Twin-Strep(II)-tag on the C terminus of APC4, was purified by affinity to Strep-Tactin Sepharose (IBA), then by ion exchange, and finally by SEC. APC/C–CDH1–EMI1–SKP1 for EM was prepared by mixing the lysate from HighFive cells expressing APC/C or Myc-6xHis-CDH1. APC/C–CDH1 was captured on Strep-Tactin Sepharose and then incubated with purified FLAG-EMI1/SKP1 (14). APC/C–CDH1–EMI1–SKP1 eluted from the Strep-Tactin Sepharose was affinity purified again using anti-FLAG M2 affinity gel (Sigma). The equivalent of 250 μg of APC/C–CDH1–EMI1–SKP1 was further processed through GraFix (37). Recombinant UBA1, UBE2C, UBE2S, CDH1, EMI1/SKP1, and ubiquitin were all expressed and purified as described previously (8, 14, 23, 38). Cyclin B NTD* (1–95) was purified and C-terminally labeled with fluorescein 5-maleimide, as denoted by the asterisk (38).

Recombinant cohesin complexes were expressed in Sf9 cells and purified essentially as described (26). Cohesin tetramers, which were expressed with a His-tag on SA1 and a Flag-tag on SMC3, were purified by affinity to Ni-NTA agarose beads (Qiagen), eluted with 150 mM imidazole, and then purified by affinity to anti-FLAG M2 affinity gel (Sigma) and eluted with 0.5 mg/mL Flag peptide.

Recombinant yeast kinetochore subcomplexes were expressed in Sf9 cells. The complexes were purified by affinity and SEC. The Ndc80 (N) complex and theMN complex were expressed with a His-tag on Ndc80 and were purified by affinity to Ni-NTA agarose beads (Qiagen). The Mis12 (M) complex was expressed with a His-tag on Dsn1 and was purified by affinity to Ni-NTA agarose beads (Qiagen). The KMN complex was expressed with a Flag-tag on Spc105 and was purified by affinity to anti-FLAG M2 agarose beads (Sigma). The Ndc80 (N) and Mis12 (M) complexes were further purified by SEC using a Superdex 200 10/300GL column (GE Healthcare). The MN and KMN complexes were further purified by SEC using a Superose 6 10/300GL column.

Protein Complex Characterization. APC/C-mediated ubiquitination assays were performed as previously described (8, 14, 23). In summary, 30 nM APC/C, 1 μM CDH1, 200 nM UBE2C, 200 nM UBE2S, 1 μM EMI1, 0.5 mg/mL BSA, 100 nM UBA1, 5 mM $MgCl_2$, 5 mM ATP, and 500 nM fluorescein-labeled $CycB^{NTD*}$ were mixed on ice. The reactions were then equilibrated to room temperature and initiated by addition of 250 μM Ub. Following a defined incubation period, the reactions were quenched and then separated by SDS/PAGE. Ubiquitinated $CycB^{NTD*}$ was visualized using a Typhoon FLA 9500 PhosphorImager. The cryo-EM structure of APC/C–CDH1–EMI1–SKP1 was determined essentially as previously described (8, 23). Recombinant cohesin ATPase assays and rotary shadowing EM were performed as previously described (26).

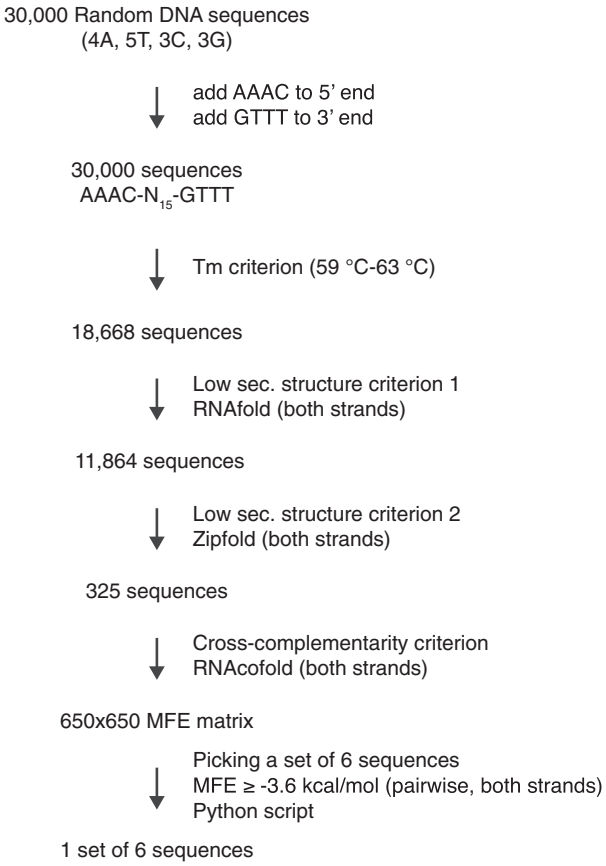

Fig. S1. Generation of optimized DNA linker sequences. To generate optimized homology sequences for a six-fragment Gibson assembly reaction, 30,000 random DNA sequences of 15 nt in length and defined base composition were generated, and the sequences AAAC and GTTT required for compatibility with PmeI digestion were added to their 5′ and 3′ ends, respectively. Melting temperatures were predicted, and only sequences within the melting temperature range of 59–63 °C were included in further analyses (18,668 sequences). Sequences with a high probability of secondary structure formation on either strand were excluded using RNAfold (11,864 sequences left). The sequence set was further analyzed for secondary structure formation with Zipfold using parameters that fit with Gibson reaction conditions. Only sequences for which both strands were very unlikely to form secondary structures in Gibson assembly reactions [minimum free energy (MFE) $\geq +1.5$ kcal/mol] remained in the sequence set (325 sequences). To pick a set of sequences that provides highest specificity in Gibson reactions (i.e., no false annealing), hybrid structures for each combination of two sequences out of the set of 325 sequences and their reverse complements were predicted using RNAcofold. Free energy values of the MFE structure of each hybrid were entered into a 650×650 MFE matrix. An algorithm was written in python to build sequence sets in which no pairwise interactions violate an MFE threshold. When setting the threshold to MFE ≥ -3.6 kcal/mol, it was possible to pick exactly one set of six sequences. This set is used as linker sequences in the second assembly step (linkers A, B, C, D, E, and F). This set was manually slightly modified to generate a set that is applicable in the first assembly step (linearization with SwaI), while maintaining all thermodynamic criteria (linkers α, β, γ, δ, ε, and ω).

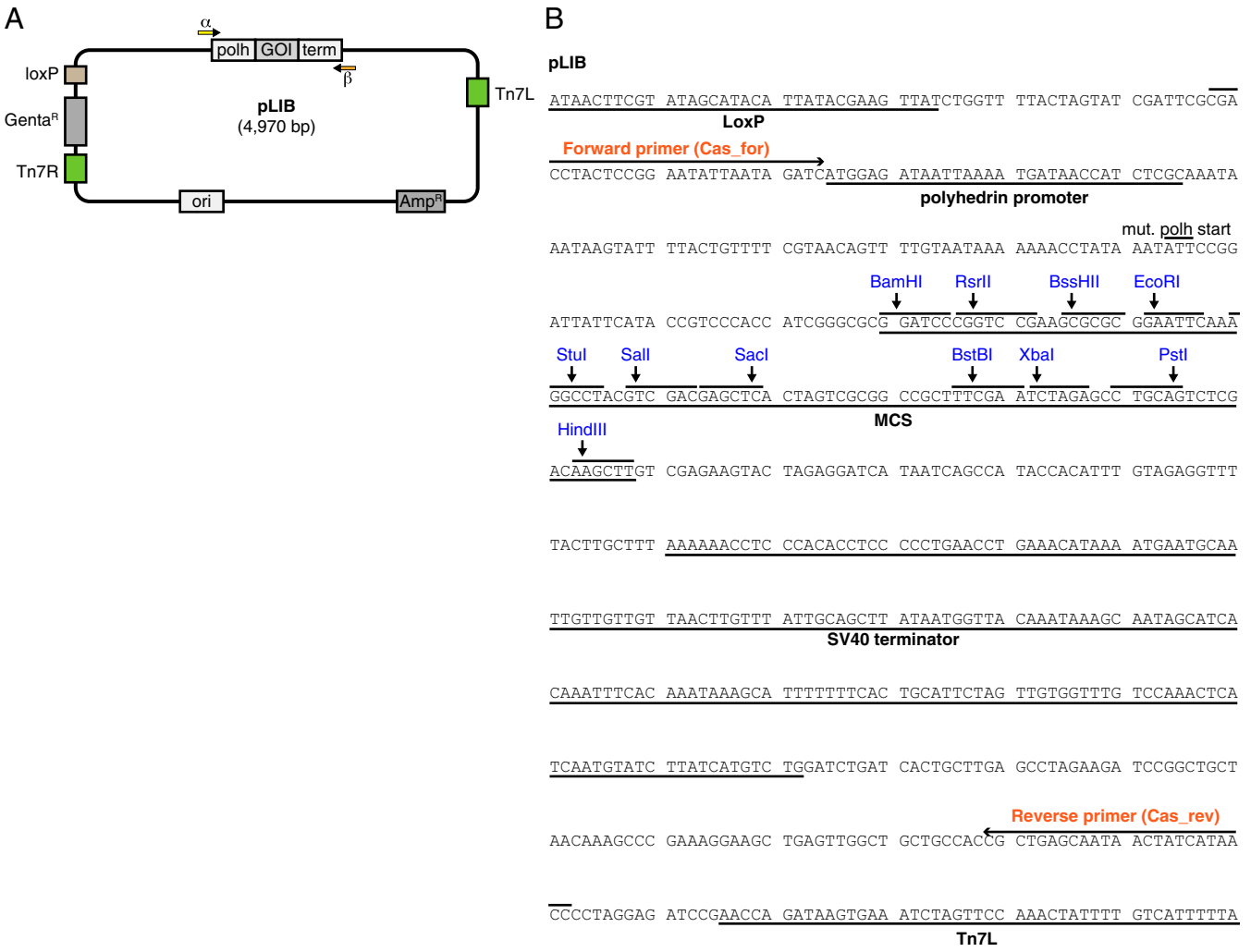

Fig. S2. pLIB vector. (*A*) Schematic representation of pLIB. The cDNA of a GOI is inserted between the polyhedrin promoter (polh) and the SV40 terminator sequence (term). pLIB can be maintained with Ampicillin (Amp^R resistance gene). All biGBac vectors (pLIB, pBIG1, pBIG2) contain Tn7 elements (Tn7L, Tn7R) and a gentamicin resistance gene (Genta^R) for generation of baculoviruses and a LoxP site for compatibility with Multibac donor plasmids. (*B*) DNA sequence of empty pLIB from LoxP site to Tn7L element. For generation of library constructs using Gibson reactions, a stock of linearized pLIB can be generated by BamHI/HindIII digestion and a cDNA can be inserted via a Gibson reaction (see *Materials and Methods* for good homology sequences). Alternatively the MCS can be used to insert a cDNA by conventional restriction/ligation cloning. It is recommended to not clone ORFs in-frame with the mutated polyhedrin start codon (mut. polh start) to avoid the possibility of leaky expression that might lead to N-terminal extensions. The binding sites of the predefined oligonucleotide set (Table S1) for amplification of GECs are indicated (Cas_for/Cas_rev).

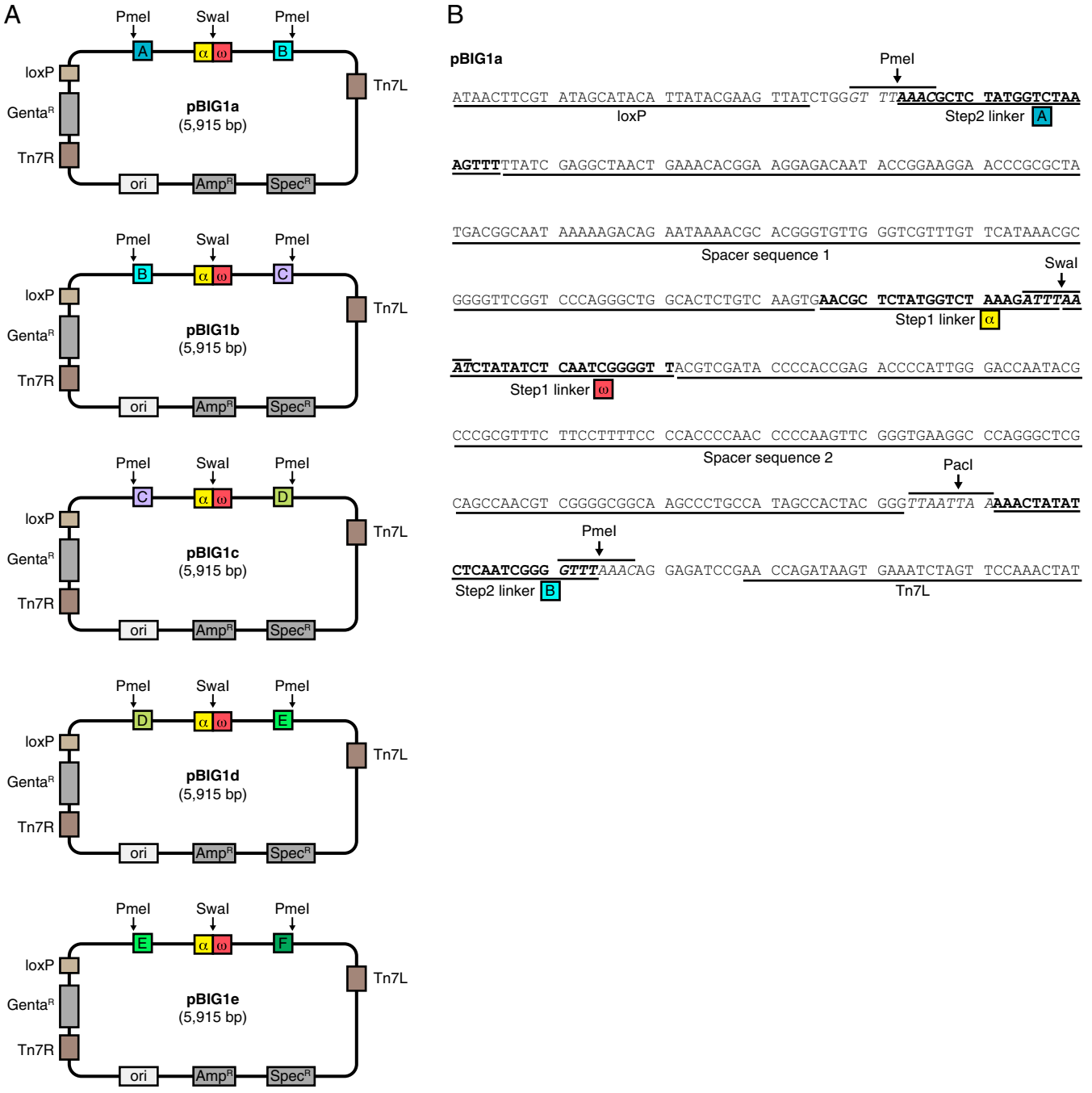

Fig. S3. pBIG1 vectors. (*A*) Schematic representation of pBIG1 vectors. pBIG1 vectors can be maintained with Spectinomycin (Spec^R resistance gene) or Ampicillin (Amp^R resistance gene). For the selection in the first assembly step, Spectinomycin is used. Stocks of linearized pBIG1 cloning vectors are generated by SwaI digestion. This results in linear vector backbone with linker sequences α and ω at the fragment ends. After the first assembly step, the generated PGCs can be released from the vector backbone by PmeI digestion. The five pBIG1 vectors differ only in the linker sequences (A, B, C, D, E, and F) next to the PmeI sites as indicated. All biGBac vectors (pLIB, pBIG1, pBIG2) contain Tn7 elements (Tn7L, Tn7R) and a Gentamicin resistance gene (Genta^R) for generation of baculoviruses and a LoxP site for compatibility with Multibac donor plasmids. (*B*) DNA sequence of pBIG1a shown from the LoxP site to Tn7L element. The positions of α, ω and A, B linker sequences as well as of the restriction sites SwaI, PmeI, and PacI are shown. The linker sequences of the first and the second assembly step are separated by spacer sequences (derived from HSVtk terminator sequence in pFL) to avoid interference of the two linker sequence sets in Gibson reactions.

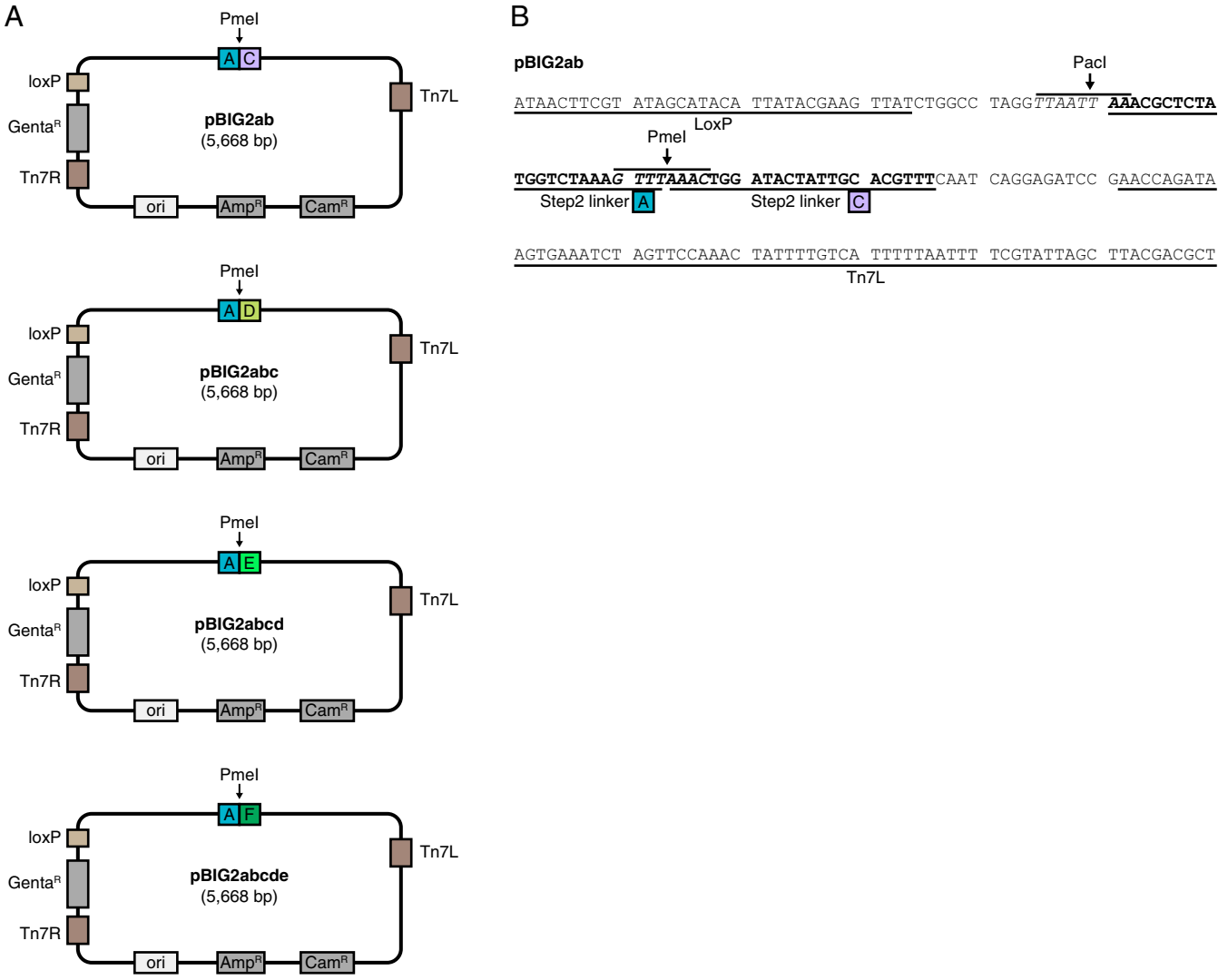

Fig. S4. pBIG2 vectors. (*A*) Schematic representation of pBIG2 vectors. pBIG2 vectors are maintained with Chloramphenicol (Cam^R resistance gene) or Ampicillin (Amp^R resistance gene). For the selection in the second assembly step, Chloramphenicol is used. Stocks of linearized pBIG2 cloning vectors are generated by PmeI digestion. This results in linear vector backbone with linker sequences on both ends. The four pBIG2 vectors contain linker sequence A on one end and differ only in the linker sequence on the other side (C, D, E, or F as indicated). All biGBac vectors (pLIB, pBIG1, pBIG2) contain Tn7 elements (Tn7L, Tn7R) and a Gentamicin resistance gene (GentaR) for generation of baculoviruses and a LoxP site for compatibility with Multibac donor plasmids. (*B*) DNA sequence of pBIG2ab shown from the LoxP site to Tn7L element. The positions of linker sequences A and C and the restriction sites PmeI and PacI are shown.

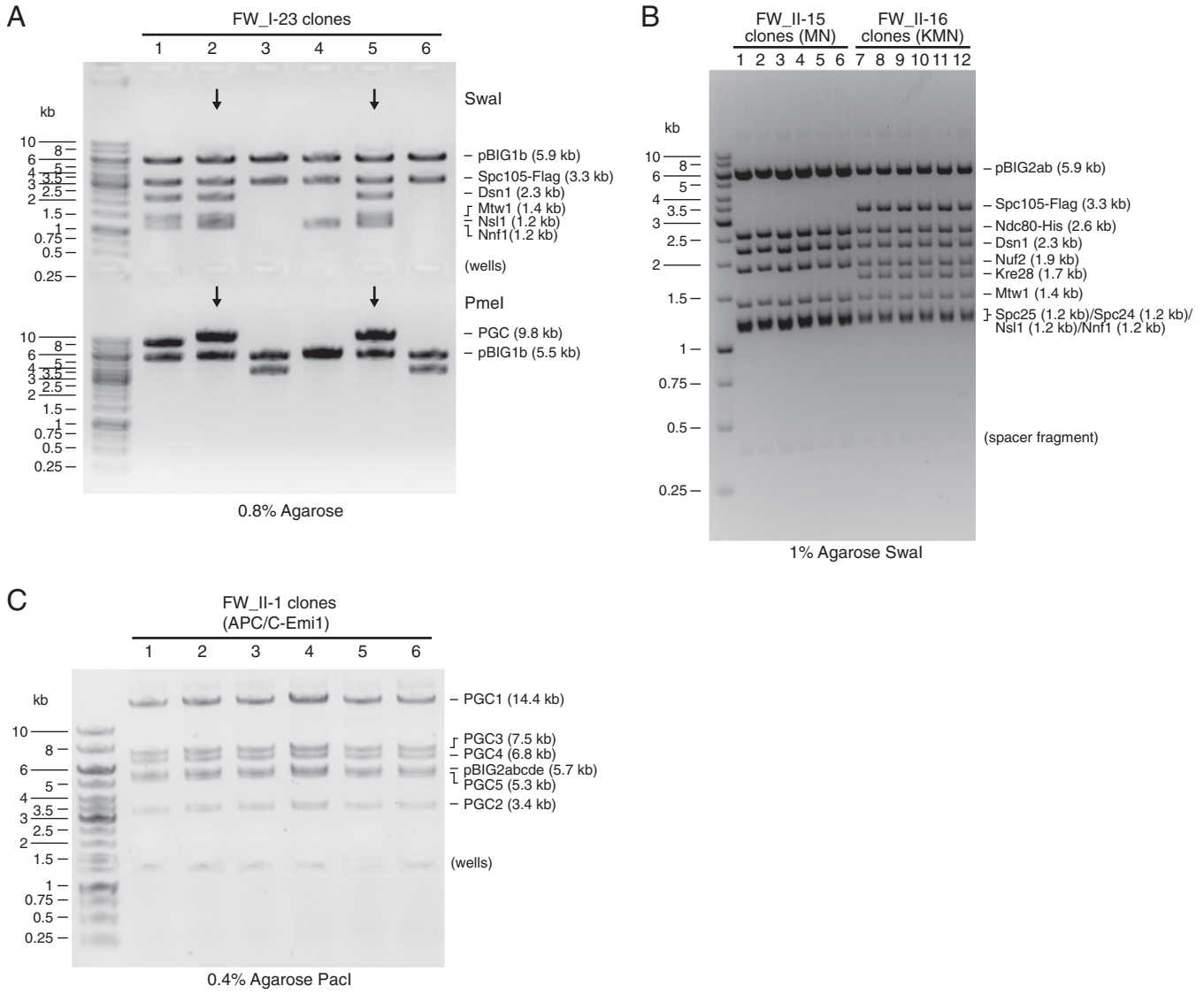

Fig. S5. Examples for construct analysis. (*A*) Analytical digest of a pBIG1 construct. Six clones of FW_I-23 (pBIG1b:Dsn1/Mtw1/Nnf1/Nsl1/Spc105-Flag) were digested by SwaI or PmeI and analyzed on a 0.8% agarose gel. The expected sizes of pBIG1 vector backbone and GECs after SwaI digestion and of pBIG1 vector backbone and the PGC after PmeI digestion are indicated. Note that spacer sequences (see Fig. S3) stay with the vector backbone after SwaI digestion (5.9 kb) and with the PGC after PmeI digestion (backbone 5.5 kb). Clones 2 and 5 show the correct restriction pattern. (*B*) Analytical SwaI digest of pBIG2 constructs. Six clones of FW_II-15 (Kinetochore MN) and six clones of FW_II-16 (Kinetochore KMN) were digested with SwaI and analyzed on a 1% agarose gel. The expected sizes of pBIG2 vector backbone and GECs are indicated. Note that SwaI digestion of pBIG2 constructs yields an additional fragment of 0.3 kb consisting mainly of spacer sequences between PGCs. All clones show the correct restriction pattern. (*C*) Analytical PacI digest of a pBIG2 construct. Six clones of FW_II-1 (APC/C-Emi1) were digested with PacI and analyzed on a 0.4% agarose gel. The expected sizes of pBIG2 vector backbone and PGCs are indicated. All clones show the correct restriction pattern.

Table S1. Linker sequences and predefined oligonucleotide set

Identifier	Sequence
Linker sequences for second assembly step	
A	AAACGCTCTATGGTCTAAAGTTT
B	AAACTATATCTCAATCGGGGTTT
C	AAACTGGATACTATTGCACGTTT
D	AAACCTAATGATGCCTGATGTTT
E	AAACGGTTCACATAGCTTAGTTT
F	AAACACTGACATTGACTTGGTTT
Linker sequences for first assembly step	
α (=mod. A)	AACGCTCTATGGTCTAAAGATTT
β (=C)	AAACTGGATACTATTGCACGTTT
γ (=D)	AAACCTAATGATGCCTGATGTTT
δ (=E)	AAACGGTTCACATAGCTTAGTTT
ε (=F)	AAACACTGACATTGACTTGGTTT
ω (=mod. B)	AAATCTATATCTCAATCGGGGTT
Predefined oligonucleotide set	
CasI_for	AACGCTCTATGGTCTAAAGATTTAAATCGACCTACTCCGGAATATTAATAGATC
CasI_rev	AAACGTGCAATAGTATCCAGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasII_for	AAACTGGATACTATTGCACGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasII_rev	AAACATCAGGCATCATTAGGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasIII_for	AAACCTAATGATGCCTGATGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasIII_rev	AAACTAAGCTATGTGAACCGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasIV_for	AAACGGTTCACATAGCTTAGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasIV_rev	AAACCAAGTCAATGTCAGTGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasV_for	AAACACTGACATTGACTTGGTTTAAATCGACCTACTCCGGAATATTAATAGATC
Casω_rev	AACCCCGATTGAGATATAGATTTATTTAAATGGTTATGATAGTTATTGCTCAGCG

Table S2. Timetable for generation of biGBac expression constructs

Day	Experimental steps	Time*
Day 1	PCR GECs	~8–10 h
	PCR "cleanup," determine DNA concentration, agarose gel electrophoresis	
	Step 1 Gibson assembly reaction	
	Transformation of *E. coli*	
Day 2	Inoculate *E. coli* overnight cultures	~20 min
Day 3	"Miniprep" DNA isolation	~6–8 h
	SwaI/PmeI digests, agarose gel electrophoresis	
	Submit for DNA Sanger sequencing	
Day 4	Analyze sequencing results	~6 h
	Step 2 PmeI digest and Gibson assembly reaction	
	Transformation of *E. coli*	
Day 5	Inoculate *E. coli* overnight cultures	~10 min
Day 6	Miniprep DNA isolation	~4 h
	SwaI/PacI digests, agarose gel electrophoresis	

*Times are estimated for the generation of five pBIG1 and one pBIG2 construct. The generation of multiple constructs in parallel can require longer periods of time.

Table S3. pBIG1 constructs in this study

pBIG1 construct	Alias	Vector	CasI	CasII	CasIII	CasIV	CasV	Construct size, kb	Shown in figure	Clones correct/analyzed
FW_I-1		pBIG1a	APC1	APC2	APC5	APC11	APC15	20.0	Fig. 2*A*	1/3
FW_I-2		pBIG1b	APC4-Strep	—	—	—	—	8.9	Fig. 2*A*	3/3
FW_I-3		pBIG1c	APC6	APC7	APC12(CDC26)	APC13	APC16	13.2	Fig. 2*A*	1/7
FW_I-4		pBIG1d	APC3	APC8	APC10	—	—	12.5	Fig. 2*A*	2/3
FW_I-5		pBIG1e	CDH1	EMI1	SKP1	—	—	10.9	Fig. 2*A*	4/12
FW_I-6		pBIG1b	APC4-Strep	APC8	—	—	—	11.4	—	2/7
FW_I-7		pBIG1c	APC6-Strep	APC7	APC12(CDC26)	APC13	APC16	13.3	—	2/13
FW_I-8		pBIG1d	APC3m	APC8	APC10	—	—	12.5	—	1/3
FW_I-9		pBIG1d	APC3	APC8m	APC10	—	—	12.5	—	1/3
FW_I-10		pBIG1d	APC3	APC8	APC10m	—	—	12.5	—	1/3
FW_I-11		pBIG1d	APC3m	APC8m	APC10	—	—	12.5	—	1/3
FW_I-12		pBIG1d	APC3m	APC8	APC10m	—	—	12.5	—	2/3
FW_I-13		pBIG1d	APC3	APC8m	APC10m	—	—	12.5	—	1/3
FW_I-14		pBIG1d	APC3m	APC8m	APC10m	—	—	12.5	—	2/3
FW_I-15		pBIG1d	APC3	APC8	—	—	—	11.3	—	2/3
FW_I-16	Cohesin wt	pBIG1c	SMC1A	SMC3-Flag	SCC1	His-SA1	—	21.2	Fig. 3*F*	1/6*
FW_I-17	Cohesin KA	pBIG1c	SMC1A(KA)	SMC3(KA)-Flag	SCC1	His-SA1	—	21.2	Fig. 3*F*	1/42*
FW_I-18	Cohesin EQ	pBIG1c	SMC1A(EQ)	SMC3(EQ)-Flag	SCC1	His-SA1	—	21.2	Fig. 3*F*	4/30*
FW_I-19	Kinetochore N	pBIG1a	Ndc80-His	Nuf2	Spc24	Spc25	—	12.9	Fig. 3*K*	2/12
FW_I-20	Kinetochore M	pBIG1b	His-Dsn1	Mtw1	Nnf1	Nsl1	—	12.0	Fig. 3*K*	1/6
FW_I-21		pBIG1b	Dsn1	Mtw1	Nnf1	Nsl1	—	12.0	—	3/6
FW_I-22		pBIG1a	Ndc80-His	Nuf2	Spc24	Spc25	Kre28	14.6	—	2/10
FW_I-23		pBIG1b	Dsn1	Mtw1	Nnf1	Nsl1	Spc105-Flag	15.3	Fig. S5*A*	2/12

*Although the assembly of APC/C and kinetochore biGBac constructs occurred with high efficiency, more transformed *E. coli* clones needed to be analyzed when we assembled the four cohesin core subunits into pBIG1 vectors, possibly due to the relatively large size of the cohesin cDNAs, resulting in pBIG1 constructs of 21.2 kb. We assume this is due to difficulties generating pure PCR products of large GECs. We therefore also generated cohesin expression vectors in two steps with the two SMC subunits on pBIG1a and SCC1 and SA1 on pBIG1b. In the second step, we combined the four subunits into a pBIG2 expression vector. Using this strategy, most analyzed clones were correct. If several subunits of a protein complex are large, it may therefore be advantageous to distribute the cDNAs encoding the large subunits onto more than one pBIG1 vector.

Table S4. pBIG2 constructs in this study

pBIG2 construct	Alias	Vector	PGC1	PGC2	PGC3	PGC4	PGC5	Construct size, kb	Shown in figure	Clones correct/analyzed
FW_II-1	APC/C–Emi1	pBIG2abcde	FW_I-1	FW_I-2	FW_I-3	FW_I-4	FW_I-5	43.6	Fig. 2*A* and Fig. S5*C*	6/6
FW_II-2	APC/C	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-4	—	38.3	Fig. 2*B*	1/4
FW_II-3	Platform	pBIG2ab	FW_I-1	FW_I-2	—	—	—	23.7	Fig. 2*B*	2/4
FW_II-4	Platform+8	pBIG2ab	FW_I-1	FW_I-6	—	—	—	26.0	Fig. 2*B*	2/4
FW_II-5	Arclamp	pBIG2abcd	pBIG1a(empty)	pBIG1b(empty)	FW_I-3	FW_I-4	—	21.0	Fig. 2*B*	1/1
FW_II-6	Arclamp(6-Strep)	pBIG2abcd	pBIG1a(empty)	pBIG1b(empty)	FW_I-7	FW_I-4	—	21.1	Fig. 2*B*	1/1
FW_II-7	APC/C(3m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-8	—	38.3	Fig. 2*B*	2/4
FW_II-8	APC/C(8m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-9	—	38.3	Fig. 2*B*	1/1
FW_II-9	APC/C(10m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-10	—	38.3	Fig. 2*B*	1/1
FW_II-10	APC/C(3/8m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-11	—	38.3	Fig. 2*B*	5/10
FW_II-11	APC/C(3/10m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-12	—	38.3	Fig. 2*B*	1/1
FW_II-12	APC/C(8/10m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-13	—	38.3	Fig. 2*B*	1/4
FW_II-13	APC/C(3/8/10m)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-14	—	38.3	Fig. 2*B*	1/1
FW_II-14	APC/C(Δ10)	pBIG2abcd	FW_I-1	FW_I-2	FW_I-3	FW_I-15	—	37.1	Fig. 2*B*	1/1
FW_II-15	Kinetochore MN	pBIG2ab	FW_I-19	FW_I-21	—	—	—	19.5	Fig. 3*K* and Fig. S5*B*	6/6
FW_II-16	Kinetochore KMN	pBIG2ab	FW_I-22	FW_I-23	—	—	—	24.5	Fig. 3*K* and Fig. S5*B*	6/6

Table S5. Advantageous features of biGBac

Simple—can be used in standard molecular biology laboratories, no robotics needed
Efficient—cloning efficiency for APC/C pBIG1 and pBIG2 on average ~33% and ~80%, respectively
Fast—final pBIG2 multigene expression constructs can be generated in 6 d
Scalable—can be used to generate up to 40 constructs in parallel
Flexible—any biGBac construct can be used for baculovirus generation (pLIB, pBIG1, pBIG2), does not rely on combinations of "donor" and "acceptor" vectors
Modular—by allowing multiple combinations of different biGBac constructs (mix and match)